Novy.

TOXINS AND ANTITOXINS.

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Mr. Chairman, Ladies and Gentlemen—It is well recognized today that all disease bacteria, and sometimes non-pathogenic bacteria produce, in addition to various harmless, fermentative products, certain chemical poisons. Let us inquire as to the nature of these poisons. An Italian toxicologist, Selmi, 20 years ago, while examining extracts from dead bodies, met with substances which, in their chemical behavior to reagents, resembled the vegetable alkaloids, such as strychnine, atropine, morphine, etc. To this class of substances formed in the body after death he gave the name ptomain or cadaveric alkaloid. Inasmuch as at that time the rôle of bacteria in putrefaction was but little understood the genesis of these products was necessarily uncertain. Though Selmi devoted the last years of his life to the study of these basic compounds, yet, owing to imperfect methods, at no time did he succeed in isolating a chemically pure product. It was Brieger, of Berlin, who succeeded, by means of new methods devised by himself, in isolating in a condition of chemical purity a large number of these basic products or ptomains from decomposing animal matter. In the course of about five years he described no less than twenty-seven distinct ptomains. Other observers have increased the number till today we can list more than sixty representatives of this group.

At first Brieger studied the ptomains formed in decomposing flesh, where a variety of different bacteria are at work. Subsequently he extended his researches to pure cultures of pathogenic bacteria, and in a short time was able to demonstrate the presence of poisonous ptomains in cultures of the typhoid bacillus, the germ of tetanus, and the comma bacillus of Asiatic cholera. Here for the first time was a definite answer as to how disease bacteria produce their results. Inasmuch as certain higher plants are known to be poisonous because of the presence of poisonous alkaloids it was assumed that the toxic properties of bacteria were due to bacterial alkaloids or ptomains. So firmly did this view take hold that even until the present time the impression prevails that the dreaded weapons by which bacteria produce disease are

ptomains. This, however, is not true; indeed it is far removed from the truth. In the first place there are disease bacteria, such as those of diphtheria and glanders, which in spite of most careful search have failed to yield ptomains. Here there are germs of the most virulent type, the poisons of which certainly do not belong to this class. Again the ptomain isolated from a culture of a pathogenic germ may be incomparably less poisonous than the original fluid from which it was prepared. As an ilustration let us take a culture of the tetanus bacillus. From it we can isolate no less than four distinct ptomains. A dose of one-half gram of tetanin, the most energetic of these four ptomains, is almost without effect in a guinea-pig. It is therefore a comparatively weak poison. On the other hand, the culture liquid from which it was obtained, deprived of all germs by filtration, is so poisonous that 1-500 of a grain of the liquid is fatal to a guinea-p'g. This fatal dose includes, besides the poisonous substance, water as well as inert matter. If we take the total solids in this liquid as amounting to 2½ per cent., and that is nearly twice as much as usual, we will have 1-20,000 of a gram of solids. In other words the liquid from which the ptomain tetanin is obtained contains solids in solution which are ten thousand times more poisonous than the ptomain itself.

Again ptomains have been isolated from cultures of disease bacteria and found to be perfectly harmless. This indeed is true of the majority of ptomains that are known. As a rule they are either not poisonous, or but slightly so. Some, it is true, are active poisons, but their power in this respect is weak compared with other products produced by that same germ. Ptomains are therefore no longer to be considered as the active poison secreted by the bacterial cell. They are but of secondary importance as factors in the causation of disease. To the chemist, and above all to the toxicologist, these products will always be of the greatest interest.

Weir Mitchell and Reichert of Philadelphia, in 1886, in the course of their study as to the nature of the venom of serpents, made a most important discovery. They found the poison of various venoms to belong to the proteid group. One of these poisons belonged to the group of peptones, another to the group of globulins. This observation, so remarkable, and at variance with the then accepted views regarding proteid substances, attracted but little attention. Two years later, however, Roux and Yersin published their classical studies on diphtheria. In this work they were able to show that the diphtheria poison was entirely different from any other known poison. In its behavior to heat, acids and other reagents it resembled ferments, such as pepsin or diastase. They therefore inclined to the belief that the diphtheria poison was a ferment—an enzyme.

This observation led Brieger and Fraenkel to re-investigate the poisons of a number of disease bacteria. By the addition of alcohol or of ammonium sulphate to the filtered bacterial cultures, precipitates were obtained, which after repeated purification gave proteid reactions, and what was more important, in exceedingly minute doses were poisonous to animals. Here, then, apparently, was the long-sought-for bacterial poison. Ptomains were but feeble poisons compared with the action of these products. Inasmuch as these substances are proteid in nature, it is customary to speak of them as bacterial proteids. Representatives of the different

groups of proteids have been isolated from cultures of various bacteria. Thus from diphtheria an albumin was obtained—the so-called toxalbumin. From cultures of the anthrax bacillus poisonous albumoses were isolated. Pure cultures of the cholera germ yielded a highly poisonous pepton and globulin.

A striking analogy was thus established between the deadly venom of serpents and equally to be feared weapon of bacteria. Another parallel was established by the discovery about the same time, that even higher plants could give rise to highly poisonous proteids. From the jequirity seed an albumose, abrin, was isolated, while from the castor bean a similar compound, ricin, was obtained. Some idea as to the intensely poisonous action of these toxic proteids may be obtained when we consider that 1-100,000 g. of abrin suffices to kill an animal weighing one kilogram. Or, in other words, 1 g. of this substance is sufficient to kill 200,000 guinea-pigs, each weighing one pound. The calculated fatal dose for a man weighing 130 pounds would be about 1-100 of a grain.

Again, Fraser has shown that 0.18 mg. (1-350 grain) of the cobra venom is fatal to a one kilogram rabbit. It is at least 16 times more powerful than the venom of the rattlesnake.

Intensely poisonous substances belonging to the proteids were therefore obtained from certain animals, namely serpents, from higher plants and from the lowest plants, namely bacteria. The products of the latter, owing to their great importance, are of course the most interesting, and have therefore been studied more diligently. The French bacteriologists have always held that the poisonous property of the bacterial proteids was not inherent in the proteid molecule, but was due to mechanical admixture of an unknown poison. Thus it was pointed out that if a precipitate of calcium phosphate or aluminum hydrate is produced in a culture medium the poison or a part of it is mechanically dragged down with the precipitate. The bacterial proteids from this standpoint are to be considered as an intimate mixture of an inert proteid and the active poison. It is possible for the proteids that are elaborated by bacteria to possess poisonous proprieties of their own, but from what is known to-day it is more likely, as in the case of the ptomains, that the real poison of our bacteria belongs elsewhere.

Brieger and Cohn in 1893 directed their attention to the isolation of the poison of the tetanus bacillus in as near a pure condition as possible. After filtering the tetanus cultures through porcelain the filterate was treated to saturation with ammonium sulphate. This throws out of solution the poison as well as proteids and other substances. After treatment with lead acetate and after dialysis to remove these various impurities, the poison was obtained as yellow, readily soluble flakes. It was so pure that it no longer gave proteid reactions. It contained no phosphorus and only unweighable quantities of sulphur. This therefore settled, at least negatively, the nature of the tetanus poison. It was not a bacterial proteid and it was not a ptomain. In its purest condition this tetanus poison was no longer precipitated by ammonium sulphate. This does not mean that the poison was chemically pure, for such probably it was not. Nevertheless, in this condition it was so poisonous that five hundred millionths of a gram was fatal to a 15-gram white mouse. To make this astounding figure a little more intelligible, we will say that a mouse weighing half an ounce is surely killed by a dose of this purified poison amounting to

1-1,128,000 of a grain. The calculated fatal dose for a man weighing 155 pounds (70 K) is 1-280 grain (0.23 mg.).

The tremendous activity of this poison is without an equal in the whole range of toxicology. It is the study of poisons of this kind that teach us, above everything else, of what fearful weapons bacteria are possessed. It is possible from this to understand how the tetanus bacillus, localized in a small wound in the body, is capable of producing most powerful effects in the entire organism.

In the diphtheria bacillus we have an organism which in its power to produce intensely poisonous products stands close to the tetanus bacillus. The poison of both of these germs is found especially in solution in the liquid in which the germs are grown. As already stated, the active agent of the diphtheria bacillus is not a ptomain. It is something else. Roux and Yersin believed it to be an enzyme or soluble ferment, whereas Brieger and Frænkel, from their work in 1890, concluded it was a toxalbumin. Is it, however, a proteid substance, an albumin? Or is the proteid substance that is precipitated from a filtered culture of the diphtheric bacillus merely a drag-net, which mechanicaly carries down with it the real poison, which itself is non-proteid? The latter is undoubtedly the case, for the poison can be thrown out of solution by the production of a precipitate of barium sulphate or calcium phosphate in the original culture lipuid. The poison, as in the case of tetanus, is precipitated by ammonium sulphate. By repeated purification it has been obtained fairly pure, so that 1 mg. (1-64 grain) suffices to kill a guinea-pig. That it is much more poisonous than this there can be no doubt. In its purest condition, as obtained recently by Brieger, it fails to give proteid reactions. Furthermore, it dialyzes quite readily through parchment paper. These facts, then, conclusively show that the diphtheria poison, like that of tetanus, is not a proteid substance.

From what has been said of the poisons of the diphtheria and tetanus germs, it is evident that soluble, intensely poisonous products are produced during the growth and multiplication of bacteria. It was formerly supposed that these poisons are formed outside of the bacterial cell, by ferment action on the nutrient substances in the culture medium. That is to say, that they are cleavage products, in the sense that albumoses and peptones are cleavage products, resulting from the action of pepsin on proteids. This view, in the light of our present knowledge, must likewise be abandoned.

The studies of the past few years have clearly shown, (1) that the bacterial poisons are not cleavage products, resulting from the breaking down of proteid matter; (2) that they are not proteids in nature, and (3) that they are not ptomains. So much for what they are not. When then is the nature of these mysterious, powerful, poisonous substances, elaborated by these wonderful microscopic forms of life? It must be confessed that at present we do not know what they are. The characteristic poison of a germ has as yet not been obtained in a condition of absolute chemical purity. An ultimate analysis is therefore impossible. The properties that these poisons do possess are so marked, so characteristic, as to leave no doubt that we have to do with an entirely new group of chemical products. Goethe says: "Denn eben wo Begriffe fehlen da stellt ein Wort zur rechten Zeit sich ein." And so it is with these products; we know nothing of their nature, but to cover this void in our knowledge, we

invent a term and call them *Toxins*. The word toxin, of course, has been used to designate poisonous substances in general, but in the case of bacteria it is given a restricted meaning. It denotes the specific poison of the germ the nature of which is wholly unknown.

As previously stated, the bacterial toxins are not cleavage products. They are not produced by analytic changes. On the contrary, they are to be considered as synthetic products, built up, elaborated from the food material furnished the germ. Synthetic changes are carried on *within* the living cell, not without. It is therefore inside of the bacterial cell that these poisons are formed. In other words, every bacterial cell is itself a poison. In order, however, that this poison shall act on a living body, it is necessary for it to pass into solution, to leave the cell wherein it was elaborated, and to diffuse outward into the surrounding medium. With some germs, notably the tetanus and diphtheria bacilli, this outward diffusion of the poison readily takes place. As a result, the liquid in which these germs are grown acquires enormous poisonous powers, owing to the soluble poison which they contain. On the other hand, there are many bacteria in which this outward diffusion or dialysis of the poison does not readily take place. This is true of the germs of cholera, typhoid fever, hog cholera, anthrax, etc. In such cases the toxin remains stored up within the cell, and it can be obtained from these only by special procedures. The filtered culture liquids in such cases are but feebly poisonous.

The anthrax bacillus, as stated, is one of those germs where the specific toxin is stored up within the cell and leaves it only under special conditions. Marmiér, for instance, has shown that the anthrax bacillus, if grown at the temperature of the body in a good nutrient medium, that is, under conditions which are the very best for the healthy growth of the germ, gave off but little of its toxin to the surrounding medium. The filtrate from such a culture is but feebly poisonous, and when examined for a toxin yields but very small amounts. On the other hand, if the same germ is grown under adverse conditions, such as a low temperature and an unfavorable soil, the filtrate became exceedingly poisonous, and on chemical examination gave a large amount of the specific toxin. It would seem that the anthrax bacillus, when grown under the healthiest conditions, retains nearly all of its toxin within the cell. The conditions are so favorable that but few of the cells die, and hence but little poison passes outward. The cells must die, disintegrate, in order that the toxin shall be found in solution in the culture liquid. This is exactly the condition that exists when it is forced to grow under unfavorable conditions. The cells are struggling for their existence, many of them die, and as a result the filtrate becomes highly toxic. These conditions may prevail in the living body. Thus, in the guinea-pig, the anthrax bacillus is found always in enormous numbers, whereas in the white rat it is often difficult to find, and yet in both cases death is the result. The explanation of this seeming paradox undoubtedly lies in the facts given. In the guinea-pig the conditions are favorable, the germ vegetates abundantly, and hence give off but little of its toxin, whereas, in the rat, it is struggling for its existence, more of the cells die, hence more of the poison passes into solution. From cultures of the anthrax bacillus a ptomain has been obtained and likewise poisonous proteids of the albumose group. But, as already stated, the ptomains and bacterial proteids are but of secondary

importance in the causation of the disease. In anthrax a much more powerful toxin has been demonstrated by Marmiér. It was obtained, as in the case of tetanus and diphtheria, in a sufficiently pure condition to show that it was not a proteid substance. In many of its properties this toxin is quite different from those already mentioned. Thus, the temperature of boiling water, which almost instantly destroys the toxins of diphtheria and tetanus, the venoms of serpents and soluble ferments, has but little action on the toxin of anthrax. By repeated injections of the toxin immunity can be conferred. This is true, indeed, of nearly all toxins.

The poisons of the germ of Asiatic cholera have likewise been studied very carefully. Brieger, nearly ten years ago, obtained the ptomains, cadaverin, putrescin and methyl guanidin from culture of the germ. Subsequently, with Fraenkel, he described a proteid poison. According to Petri the poison of the cholera germ is a pepton and a similar pepton, though much more poisonous, was isolated by Scholl. It is highly probable, however, that in both these cases the pepton contained as a mechanical admixture the specific toxin, which is itself non-proteid. Brieger has lately shown that it is not precipitated by ammonium sulphate, which fact is true of the purified toxins already mentioned, and moreover, that it does not give the biuret reaction. The cholera toxin, like that of anthrax, is retained within the cell under ordinary conditions. The filtrates from cultures of the germ possess only a weak poisonous action. The toxin is a very delicate substance, readily converted by apparently harmless chemical manipulations into secondary products, which may likewise be poisonous, though to a less degree than the original toxin.

The bacillus of typhoid fever likewise produces and stores up its toxin within the cell. So far as the chemical study of this germ is concerned, we may say that it has yielded results similar to those of the cholera and tetanus germs. At first a poisonous ptomain, typho-toxin, was described; subsequently a poisonous proteid was met with. This, as in the preceding instance, is probably but a mixture of an inert proteid with the real specific toxin. The colon bacillus, which is so difficult to distinguish from the typhoid germ, gives products which must be quite similar to those of the typhoid germ as immunity experiments with these two germs have shown.

The poison of the tubercle bacillus is likewise primarily stored up within the cell, as the experiments of Prudden and Hodenpyl clearly indicate. With dead tubercle, thoroughly washed to remove all traces of soluble products, they were able to induce in animals pathological changes similar to those produced in the disease. When the tubercle bacillus is grown artificially on liquid media for some time, more or less of the active toxin passes into solution. This liquid filtered and concentrated, is what is known as tuberculin. A poisonous ptomain and a proteid of the albumose group has been found in this liquid, but the specific toxin is for the most part still unknown.

In general, we may say, that the toxins produced by bacteria are exceedingly unstable compound. A chemist may start out with a very poisonous liquid and long before he is through with the necessary chemical manipulations only inert products remain. Let us inquire briefly into the characteristic properties of these toxins. Sunlight, and even ordinary diffuse daylight, possesses a marked destructive action on these substances. A highly poisonous tetanus filtrate exposed to the sunlight for

a few hours becomes innocuous. This is also true of the diphtheria toxin, and indeed of all the bacterial toxins. It is desirable, therefore, to keep such solutions in the dark. Heat possesses even a more marked action. Toxins, as a rule, are very sensitive even to moderately high temperatures. Boiling usually promptly destroys these poisons. The tetanus toxin is so sensitive to heat that at 65° C. it is destroyed in five minutes.

Dilute acids and alkalies likewise exert marked destructive action. Thus a half per cent. hydrochloric acid within an hour destroys the tetanus toxin. Hypochlorites readily destroy the toxins. Even alcohol on prolonged contact tends to convert these substances into inert products. Furthermore, the toxins possess an exceedingly important property, with reference to the production of immunity. Repeated injections with gradually increasing doses of the crude toxin or filtrate establish in time in the body a condition of immunity. As is well known, the horse is immunized against diphtheria in the preparation of antitoxin by repeated injections of the filtered diphtheria culture. Some have assumed that bacteria gave rise to two groups of products, an immunizing substance and the toxin. This supposition, however, is not necessary since, with purified toxins, immunity can be induced.

We cannot leave the subject of bacterial toxins without pointing out the remarkably close resemblance that these products bear to the venom of serpents and to the so-called poisonous plant proteids. The most active venom is rendered inert by heat, even considerably below the boiling point. Acids, alkaline hypochlorites, gold chloride, iodine, etc., soon destroy the poisonous property. Introduced into the stomach, all three of these poisons are comparatively harmless. A quantity, a hundred times greater than the amount necessary to kill instantaneously, must be given by the mouth in order to produce fatal results. Immunity to all bacterial toxins, venoms and plant proteids, can be established by essentially the same method of experimentation. The blood of the animals thus immunized, possesses antitoxic properties. The antitoxins of diphtheria, tetanus, streptococcus, etc., are well known. Similarly, a horse immunized against the venom of a serpent yields an antitoxic blood serum, which protects not only against the venom employed, but against all other venoms.

This last point is one of great importance in the study of immunity. Closely-related organisms give rise to closely-related, if not identical, chemical products. The venom of the rattlesnake, therefore, though many times less active than that of the cobra, is, nevertheless, generically alike. It is because of this similarity and chemical relationship between the active poisonous constituents of the venom that it becomes possible to have one antitoxic blood serum which will do equally well for the cobra, rattlesnake and viper venom.

Among our bacteria, however, there is, as a rule, no such real relationship and consequently there is no close relationship between the respective toxins of various disease germs. We are accustomed to classify bacteria upon most arbitrary grounds, the mere external form of the organism. It is evident that while a bacillus may resemble another bacillus in form and size, after all, the two are wholly unlike generically. This, indeed, is more often the case. For this reason we have a specific toxin of diphtheria, a specific toxin of tetanus, another of anthrax, and still another of typhoid fever. These toxins may

possess similar reactions, but chemically and physiologically their relationship is very remote. It follows, therefore, that we must have specific antitoxins to cope with each one of these bacterial poisons. The diphtheria antitoxin acts only against the diphtheria poison, not against tetanus or anthrax. In the same way the tetanus antitoxin is useful only in tetanus, not in diphtheria.

What are antitoxins? where do they come from? and how do they act? are questions of great interest. We are thoroughly familiar with what an antitoxin is capable of doing, but when we attempt to answer these questions we find ourselves largely in the field of speculation. We have seen how little is known regarding the true nature of the bacterial poisons. We know even less regarding the nature of antitoxins. As to their source, it is safe to say that they are products given off by certain cells of the body under the influence of the bacterial poison. The primary constituent of the nuclei of cells is an exceedingly complex body known as nucleohiston. This substance readily decomposes into nuclein and into histon. These two products possess antagonistic powers. Thus, nuclein hastens the coagulation of blood, whereas histon prevents this change.

Antitoxin was supposed by Behring to act strictly as a neutralizing agent, in the same way as an acid neutralizes an alkali. But Buchner and Roux have advanced proofs which go to show that such is not the case. Antitoxins do not neutralize directly the bacterial toxin. They do, however, stimulate the cells of the body in some way so that these take up the struggle and carry it to a successful close by destroying the germ and rendering the poison inert.

DISCUSSION.

President Wells—Theories on the action of microorganisms like most others must, as did the knights of the middle ages, always hold themselves ready to be challenged. I had, like most of you, I suppose, been led to believe before listening to this important paper of Dr. Novy that the poisons which produced the various communicable diseases were the results of the action of pathogenic organisms upon the tissues of the body, spoken of usually as ptomains. This theory seems to have been overthrown. I am glad to know this fact, and to learn the present belief concerning these poisons. Discussion of this subject is next upon our program. If no one cares to discuss the paper, perhaps there may be those present who would like to ask Dr. Novy questions concerning the subject.

Mr. Hinds—I would like to ask Professor Novy the extent of the paralysis of these animals (referring to two guinea-pigs used by Professor Novy to illustrate the effect of antitoxin)?

Dr. Novy—They are entirely paralyzed.

Mr. Hinds—How much of the fluid was administered?

Dr. Novy—Three drops ten days ago.

Mr. Hinds—From what will the animals die?

Dr. Novy—Heart failure.

Dr. Hutchins—I would like to have Doctor Novy explain the nature of nucleohiston.

Dr. Novy—Nucleohiston is a very complex proteid substance present in the nuclei of cells. On decomposition it yields nuclein and histon. The latter is a peptone-like body.